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Effect of Peptone on *Azotobacter* Morphology

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Pleomorphism in cultures of *Azotobacter vinelandii* was induced by addition of Difco peptone to the growth medium. Under the conditions of the study, 5% peptone gave rise to transient forms described as "fungoid cells" which were osmotically fragile. After some 48 hr of culture, they became osmotically stable and resumed a more typical morphology. It was shown that the pleomorphism-inducing principle in peptone was glycine.

Pleomorphism in bacterial cultures is generally associated with senescence, accumulation of storage materials, or the presence of specific chemical substances in the culture medium. There are various situations in which pleomorphism is the result of special circumstances such as the root nodule culture of *Rhizobium* and the growth of *Azotobacter* in nitrogen-rich media.

Although many, if not all, bacteria exhibit pleomorphism under certain conditions of culture, some species or genera show a great tendency toward the pleomorphic state. In the *Azotobacteraceae* this tendency is so great that it is, in the final analysis, difficult to ascertain the typical morphology of these organisms (12). Löhnis and Smith (8), Bisset and Hale (1), Bisset et al. (2), Winogradsky (16), and Vela et al. (13) have shown that there are many morphological variations in azotobacter cultures known to be pure, or even in those said to be clonal. Indeed, Löhnis and Smith (8) and, more recently, Bisset and Hale (1) claimed that morphological variation in pure culture represents complex life cycles of the organism, including large nonsporeforming rods, coccoid budding cells, dwarf cells, giant cells, branching cells, gram-negative rods, gram-positive rods, arthrospores, cysts, microcysts, gonidia, and spores. Although claims of morphological variation of this magnitude have been largely negated (7), it is still an unexplained fact that a single clone of *Azotobacter vinelandii* will yield at least several morphological types (2, 9). The nature of this variation has not been examined adequately and, consequently, the nature of the "true" or characteristic morphology of the azotobacter has not been described definitively.

It has long been assumed that the characteristic morphology of *Azotobacter* is that seen in young cultures in nitrogen-free media. Because azotobacter are normally found in soil and water in nature, it may well be that this represents a "laboratory morphology" only and that it is simply a manifestation of a potential rather than a characteristic (14). When *Azotobacter vinelandii* is grown in nitrogen-free media, the cells appear as generally described (3), but this is only a transient stage, and as cultures age they become pleomorphic. Many different types of cells appear in cultures after the third or fourth day of incubation. This phenomenon is so striking that the neophyte is likely to assume that his cultures have become contaminated.

Another aspect of this phenomenon is the induction of "fungoid" cells by peptone. The addition of certain concentrations of peptone to the growth medium will produce a specific type of pleomorphism characterized by fungoid forms in cultures of *Azotobacter vinelandii*. We assumed that understanding the effect of peptone on morphology would lead to some understanding of the nature of pleomorphism in *Azotobacter*.

MATERIALS AND METHODS

Cultures. *Azotobacter vinelandii* 12837 was grown at 28 C on a rotary shaker. The basic culture medium (basal medium) was Burk's nitrogen-free salts solution (15) which contained 1% (w/v) glucose autoclaved separately and peptone, amino acids, or other ingredients as indicated below. Peptone (Difco Laboratories, Inc., Detroit, Mich.) was used in most experiments and the amino acids were pure and designated grade A by the supplier (Calbiochem, Los Angeles, Calif.). Solid culture media were prepared by adding 20 g of Difco agar per liter of the desired liquid medium.

Assay for pleomorphism. The presence of fungoid cells was determined by phase-contrast microscopy. Fungoid forms were those which were 5 μm or more in length and which, at given times during the growth cycle, became swollen or spindle-shaped and divided by branching, budding, or fragmentation. The ability of peptone or its constitutents to induce pleomorphism was measured by adding the substance in question to a given volume of basal medium and inoculating with approximately 7×10^5 "normal" (nonfungoid) washed cells obtained from a culture in early log phase. These cultures, with a final volume of 1 ml, were examined at regular intervals and scored + or −, indicating pleomorphism or the lack of it. Controls were the basal medium in which pleomorphism in the form of fungoid cells did not appear and the basal medium with 5% (w/v) peptone in which pleomorphism was plainly evident within 18 hr. Relative concentrations of substances capable of inducing pleomorphism were established by making dilution series and expressing relative strength in terms of the highest dilution which would convert 1% of the normal cells to the fungoid state. This semi-quantification of fungoid-inducing substances was recorded in arbitrary units.

Fractionation of peptone. A 5% (w/v) aqueous solution of peptone was fractionated as described in Fig. 1. All fractions, residues, etc. were added to basal medium to reflect a final 5% (w/v) concentration of peptone or the amount of the given constituent in that much peptone. The *p*H of all final mixtures of peptone constituents and basal medium was adjusted to 7.0.

Chromatographic separation. A 5% (w/v) aqueous solution of peptone, which had been treated as shown in Fig. 1, was passed through an Amicon ultrafiltration cell, model 52 (Amicon Corp., Lexington, Mass.) equipped with a UM-2 Diaflo membrane in order to separate the constituents of molecular weight of less than 1,000 from larger molecules. The filtrate was concentrated 10-fold, and 1 ml was added to a Sephadex G-15 column (2 by 25 cm). The sample was eluted with water at a flow rate of 0.9 ml/min. Fractions of 1 ml were collected and tested for their capacity to induce pleomorphism.

Osmotic shock. Cultures in various liquid media were adjusted to a cell density of approximately 5×10^7/ml. Portions of 5 ml each were centrifuged, the supernatant fluids discarded, and the cells suspended in 5 ml of distilled water. Controls were suspended in their own supernatant fluid or in new growth medium. These cell suspensions were allowed to stand at room temperature (ca. 26 C) for 5 min, and then viable cell counts were determined by using basal medium plates. Microscopy examinations of all cell suspensions also were made in order to verify viable cell counts.

Amino acid analyses. The amino acid composition of several solutions was determined with a Beckman 120-C amino acid analyzer (Beckman Instruments, Inc., Palo Alto, Calif.).

Electron microscopy. Electron micrographs were obtained as previously described (13).

RESULTS AND DISCUSSION

The effect of peptone on the growth of *A. vinelandii* 12837 is shown in Fig. 2. The effect of peptone and other nitrogenous substances on azotobacter growth has been reported previously (4, 12, 16), but growth kinetics and cell morphologies were not described fully. The concentrations of peptone which allow growth (Fig. 2) induced a pleomorphism which became evident during the lag phase and most pronounced during early log growth when virtually 100% of the cells were affected. Difco peptone induced the kinds of cells called "fungoid forms" with predictable regularity. The cells shown in Fig. 3 are typical, but not all of the other morphologic types described by pre-

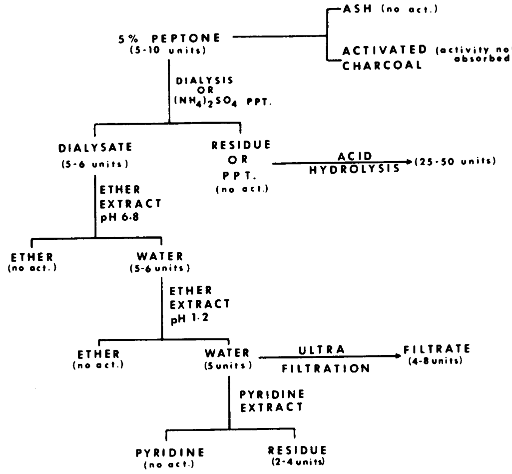

FIG. 1. *Fractionation of peptone. Figures in parenthesis indicate relative concentrations of fungoid-inducing principle in the various fractions.*

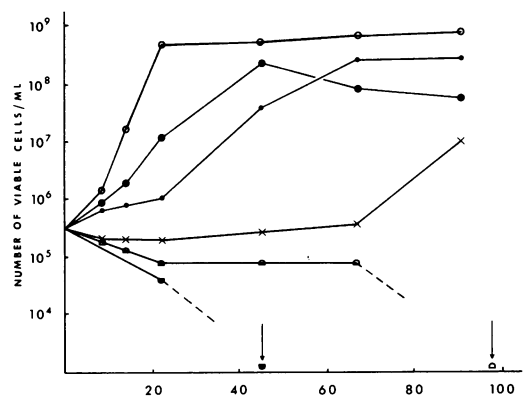

FIG. 2. *Effect of peptone added to the basal medium on the growth of A. vinelandii; arrows indicate fewer than 10^3 viable cells per ml of culture, and the following symbols indicate the amount (in percent) of peptone added to the basal medium:* ○, 0; ●, 2; •, 5; ×, 10; △, 20; *and* ▼, 30. *All values represent replicate determinations.*

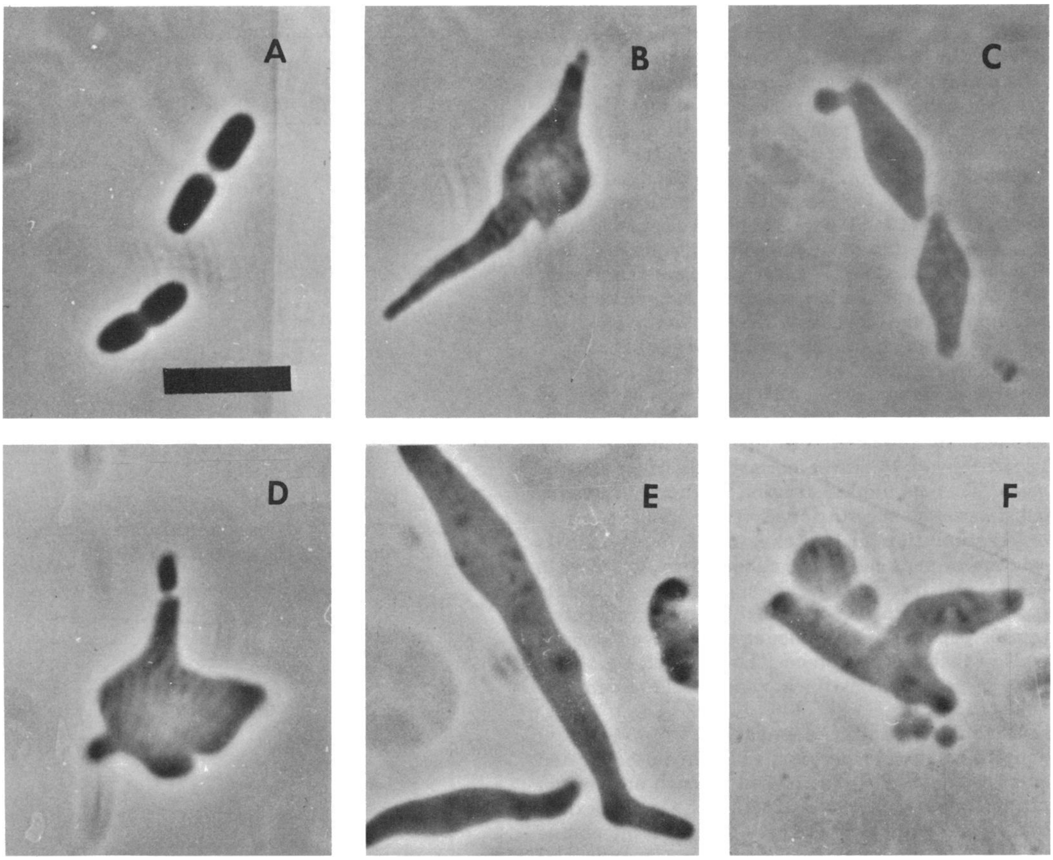

FIG. 3. *Phase contrast photomicrographs of A. vinelandii grown in basal media (A) and in basal media plus 5% (w/v) Difco peptone (B, C, D, E, F). The bar represents 4 μm.*

vious authors (1, 2, 8, 9, 12) were evident in these cultures. Under the conditions of the experiment, the morphology of the organism was determined by the presence of peptone, and the cultures could be described as pleomorphic with regard to one type or family of aberrant cells. During the phase of accelerated growth, fungoid cells divided by budding or fragmenting, and the resultant progeny reverted to a morphology more similar to that of cells grown in basal media after 30 to 48 hr of incubation. At this time, cells from both control and treated cultures were spherical or ellipsoidal in shape and frequently contained large vacuoles. In some instances, fungoid cells became filamentous and fragmented into smaller cells of morphology similar to cells grown in the basal medium. In other cases, it appeared as if the daughter cells emerged from within the fungoid cell. Lysing fungoid cells presented a classical picture of parent cells releasing "gonidia" (8), but light and electron microscopy of these cells indicated that they were mature vegetative cells of *Azotobacter.*

We infer from these data that a specific type of pleomorphism can be induced by the addition of peptone to the growth medium and that this reaction represents a unique opportunity for the study of pleomorphism. In addition to this, the data in Table 1 show why the phenomenon had probably not been described previously. It is obvious that only Difco peptone induces fungoid cell production and only over a narrow concentration range. It was not deemed necessary to the success of these studies to test other concentrations of the other peptones.

Phase-contrast microscopy of cells grown in nitrogen-free media (Fig. 3) and in the same media with 5% peptone shows that pleomorphism is pronounced but that it is limited to a recognizable set of morphological configurations. The effect of peptone on the morphology of these organisms was confirmed by

TABLE 1. *The effect of various peptones on the morphology of A. vinelandii 12837*[a]

Peptone	Concn (%)	Effect
Peptone (Difco; 3 different lots)	0.5	No effect
Peptone (Difco; 3 different lots)	1	Very slightly lengthened lag period; some cells slightly elongated and swollen.
Peptone (Difco; 3 different lots)	5	Lengthened lag period; almost all cells in culture in fungoid form after 18 to 24 hr.
Peptone (Difco; 3 different lots)	10	Same as above but growth very scanty; cells hard to classify because of low numbers.
Phytone (BBL lot 505621)	5	Little effect on lag period; no fungoid cells produced; other forms of pleomorphism evident but only to a very slight degree.
Proteose peptone (Difco lot 489565)	5	Same
Proteose peptone no. 3 (Difco lot 512435)	5	Same
Casamino Acids (Difco lot 549478)	0.5	Same
Tryptone (Difco lot 480535)	5	Same

[a] All cultures were studied simultaneously for a period of 10 days and all media were prepared by adding peptone to the same batch of basal medium.

electron microscopy (Fig. 4). Fungoid cells have an irregular form and appear to lack structural rigidity, although the structures labeled CW in the fungoid cells shown in Fig. 4 seem identical to the cell wall of the cells grown in basal media. Structural integrity was compared with that of the cells grown in basal media by osmotic shock. The data shown in Table 2 indicate that the peptone-grown cells were osmotically fragile by comparison, and we infer from this that fungoid morphology results from loss of structural integrity of the cell wall. In view of the size of fungoid cells (Fig. 3 and 4), we assumed that septum formation and subsequent division were inhibited. This assumption was based on the observation of elongated, budding, and fragmenting cells in cultures containing 5% peptone but not in cultures grown in the basal medium. These results agree with previous observations (1) of the effect of peptone on *Azotobacter*, but the number of "aberrant types" observed was limited to the fungoid forms in our experiments. We assumed that this restriction of pleomorphism was due to the specific constituents of the peptone employed. On this basis, we further assumed that we could isolate and identify the constituent(s) of peptone responsible for formation of fungoid forms in *A. vinelandii* 12837. Fractionation of an aqueous solution of peptone by the scheme indicated in Fig. 1 showed that the fungoid-inducing activity could be detected and measured during various stages of isolation. Ultrafiltration indicated that the active principle had a molecular weight of less than 1,000 daltons. Chromatographic separation of the peptone indicated that the activity was present in six 1-ml samples of a total column eluate of 50 1-ml fractions. From these data, it seemed probable that the active principle was associated with the amino acid fraction of peptone. Nondialyzable residue and $(NH_4)_2SO_4$ precipitates, both of which lacked the capacity to induce pleomorphism (Fig. 1), were hydrolyzed with 6 N HCl at 110 C for 24 hr and then tested for ability to induce pleomorphism. The activity of the hydrolysates was approximately five times greater than that of 5% peptone (Fig. 1). Amino-acid analyses of several active fractions are shown in Table 3. The values obtained from the dialysate or the ultrafiltrate were assumed to be representative of free amino acid content of aqueous solutions of 5% peptone, whereas those obtained from hydrolysates of $(NH_4)_2SO_4$ precipitates or nondialyzable material were thought to represent amino acid content of residual proteins.

Media were prepared by adding either a single amino acid or a combination of amino acids to the basal medium according to the free amino acid composition of 5% peptone. The results of these tests gave unambiguous proof that the fungoid-inducing component of peptone was glycine. Normal growth and cell morphology were observed in all cultures containing single amino acids except in those containing 300 mg of glycine per liter; in these no growth was observed. However, when 50 to 75 mg of glycine per liter were added, virtually 100% fungoid cells were observed after 24 hr of incubation. If all the amino acids shown in Table 3, except glycine, were added to the growth medium in the concentrations indicated, no fungoid forms were produced. But if 300 mg of glycine per liter were also added,

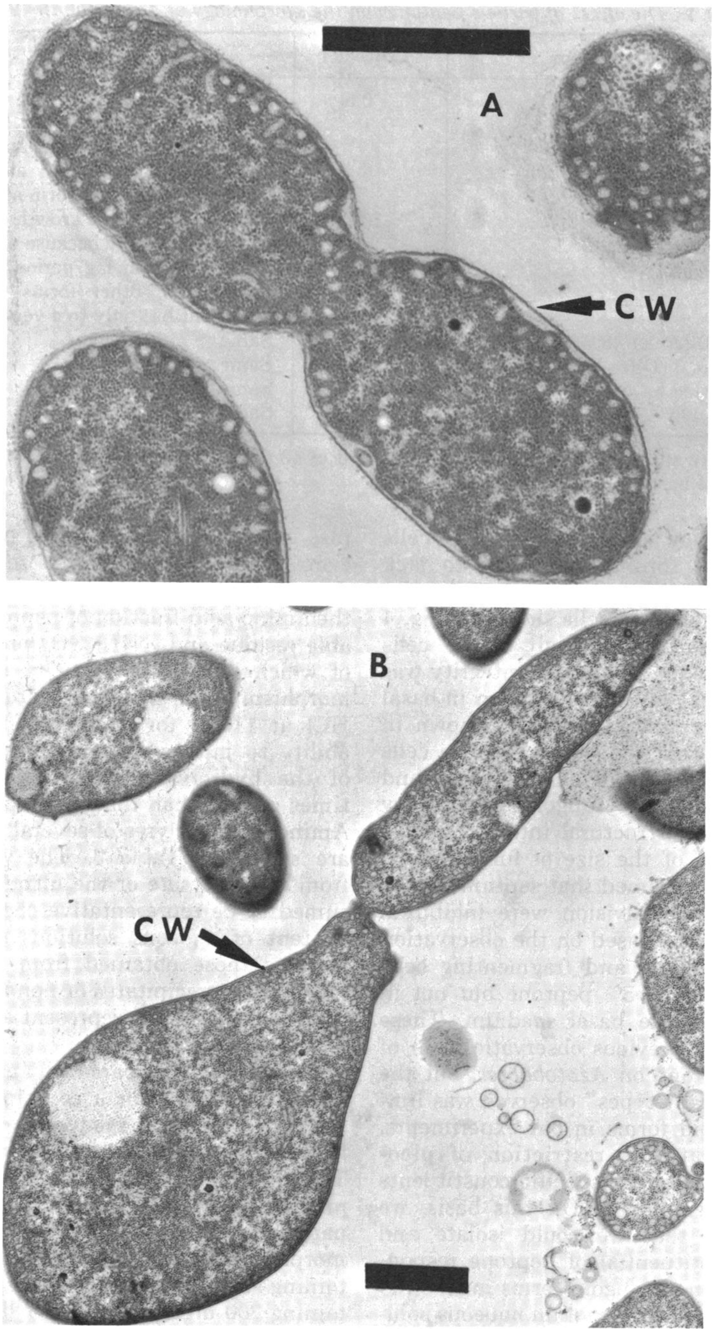

FIG. 4. *Electron micrographs of A. vinelandii grown in basal media (A) and in basal media with 5% (w/v) Difco peptone (B). Cell walls (CW) are evident in cells grown in both media. The bars represent 1 μm.*

then fungoid forms were produced. As in previous studies, fungoid cells reverted to the morphology of cells grown in nitrogen-free media in approximately 48 hr. From the above observations, it is apparent that although glycine is the sole constituent of peptone which induces fungoid formation in cells of *A. vinelandii*, its effect is influenced by other amino acids in the medium. The effect of glycine on the morphology of bacteria is well established

TABLE 2. *Comparison of osmotic fragility of cells grown in basal media and in basal media plus peptone*

Growth medium	Cell type	Survival[a]
Basal medium	Log phase	1.00
Basal medium	Stationary phase	1.00
Basal medium plus 5% peptone	Log phase	0.30
Basal medium plus 5% peptone	Late log phase	0.61
Basal medium plus 5% peptone	Stationary phase	0.80

[a] Ratio of viable cell counts after suspension for 5 min in distilled water and suspension for 5 min in the growth medium.

TABLE 3. *Amino acid analyses of various fractions of peptone*[a]

Amino acid	Membrane dialysate or ultrafiltrate	Active fractions pooled from Sephadex separation	$(NH_4)_2SO_4$ Precipitate hydrolyzed with HCl
Lys	420	Trace	170
His	100	30	40
Arg	920	180	220
Trp	100	ND	40
Asp	190	70	410
Thr	200	280	130
Ser	250	430	230
Glu	370	90	630
Pro	130	180	1,110
Gly	300	660	1,560
Ala	420	790	560
Val	360	540	200
Met	160	110	90
Ile	270	490	120
Leu	700	1,290	170
Tyr	270	ND	40
Phe	420	ND	120

[a] Milligrams per liter of 5% solution of Difco peptone. ND = none detected.

(5, 6, 10, 11) but our results (Table 4) indicate that the effect of glycine on *Azotobacter* is very critically dependent upon the concentration employed. Fungoid cells appeared predictably when 75 mg of glycine per liter was added to the medium, but there was little or no effect from half of this concentration, and four times as much inhibited growth completely.

Undoubtedly, glycine alone cannot account for pleomorphism in *Azotobacter*, but it is evident that the presence of glycine in the growth medium accounts for an important family of

TABLE 4. *The effect of glycine on the morphology and growth of Azotobacter vinelandii*

Glycine (mg/liter)	Effect[a]
3	None
30	None
38	Growth normal; cells slightly elongated; no true fungoid cells noted.
50	Lag period lengthened; fungoid cells observed during first day of culture.
75	Same
150	Growth greatly inhibited; never got turbid; a few fungoid cells observed.
300	No growth

[a] Deviation from morphology and growth rate observed in control cultures; checked daily for 2 weeks.

morphological types. Giant cells, filamentous forms, budding and branching cells, and gonidia-like structures, as described by previous investigators (1, 4, 8, 12, 16), were readily observed in cultures containing peptone or glycine.

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